First Report of *Pseudomonas* Grapevine Bunch Rot Caused by *Pseudomonas syringae* pv. *syringae*

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Abstract

Pseudomonas syringae pv. *syringae*, a Gammaproteobacterium belonging to genomospecies 2 within the *P. syringae* complex, is distributed worldwide, and it is responsible for bacterial canker on >100 different hosts, including the grapevine. *P. syringae* pv. *syringae* induces necrotic lesions in the leaf blades, veins, petioles, shoots, rachis, and tendrils on grapevine cultivars in different areas. *P. syringae* pv. *syringae* has been associated with severe economic losses in different grape cultivars in Australia, where it causes inflorescence rot. In midsummer to late summer 2017, symptoms of berry rots differing from those caused by the common berry rots agents were observed in different cultivar

Red Globe vineyards of Apulia (southern Italy). As proven by fulfillment of Koch's postulates, these symptoms were caused by a bacterium that, according to the results of biochemical, physiological, nutritional, antimicrobial activity, and pathogenicity tests and sequencing of 16S ribosomal DNA, *gyrB*, *rpoB*, and *rpoD* genes, was identified as *P. syringae* pv. *syringae*. This is the first report of *Pseudomonas* grapevine bunch rot.

Keywords: *Bacillus megaterium* syringomycin, GATTa tests, *Geotrichum candidum*, LOPAT test, syringoceptin

Pseudomonas syringae is probably the most extensively studied bacterial plant pathogen. It occurs worldwide, comprises strains isolated from >200 cultivated and wild plants (Mohr et al. 2008), and is responsible for symptoms ranging from foliar spots to blights, stripes, and cankers. On the basis of visual symptoms (Bull et al. 2010); host range (Baltrus et al. 2011); biochemical, physiological, and nutritional tests (Lelliott et al. 1966); taxonomic analysis (Young 2010); and molecular typing, the *P. syringae* complex is subdivided into 57 pathovars (Bull et al. 2010). In addition, genome-wide comparative analysis between pathovars has expanded the information on bacterial evolution and host specificity (Feil et al. 2005; Marcelletti et al. 2011). Pyoverdinin production is generally the first step in the identification of plant pathogenic *Pseudomonas* spp. (Bultreys and Kaluzna 2010; Gilbert et al. 2009; Lelliott and Stead 1987; Whitelaw-Weckert et al. 2011). Levan, oxidase, potato soft rot, arginine dehydrolase, tobacco leaf hypersensitivity reaction (LOPAT) tests and the assays based on gelatin liquefaction, aesculin hydrolase, tyrosinase activity, and tartaric acid utilization (GATTa) tests (Gašić et al. 2012; Jones 1971; Latorre and Jones 1979; Lelliott et al. 1966) are currently used to discriminate *P. syringae* from other fluorescent *Pseudomonas* species as well as the *P. syringae* pv. *syringae* from the other pathovars.

P. syringae pv. *syringae* is a Gammaproteobacterium belonging to genomospecies 2 within the *P. syringae* complex (Baltrus et al. 2011); it has a worldwide distribution (<https://www.cabi.org/isc/datasheet/45014#B97A5089-5C15-4FA9-B3E4-B61E3DF9A535>)

and is responsible for bacterial canker on a large number of horticultural and woody hosts (Bultreys and Kaluzna 2010; Golzar and Cother 2008), including stone fruits (Abbasi et al. 2013), apple (Mansvelt and Hattingh 1989), pear (Moragrega et al. 2003), and olive (Scortichini 1997). The first report of *P. syringae* pv. *syringae* in grapevine dates to 1968 (Klingner et al. 1976) in the province of Mendoza, Argentina on cultivar Cereza, and it caused symptoms such as necrotic lesions in leaf blades, veins, petioles, shoots, rachis, and tendrils. Long considered a weak pathogen, *P. syringae* pv. *syringae* has been associated with inflorescence rot, causing severe economic losses on cultivars Sauvignon Blanc, Pinot Noir, Chardonnay, and Riesling in Australia (Hall et al. 2016; Whitelaw-Weckert et al. 2011) and Kerch peninsula in Russia (Porotikova et al. 2017).

During midsummer to late summer of 2017, previously unreported soft and watery spots on berries were observed in different cultivar Red Globe vineyards of Apulia (southern Italy). Similar symptoms were also observed on cultivar VRG1, a new grapevine selection currently under trial in two experimental vineyards located in the countryside of Noicattaro (province of Bari, Apulia). This study was aimed at identifying and characterizing the pathogens responsible for the symptoms observed by means of molecular, biochemical, pathological, and microbiological methods.

Materials and Methods

Sample collection and isolation. Isolations were made from 20 symptomatic and 20 asymptomatic berries sampled from at least 10 different bunches in each of three different vineyards of table grape Red Globe and one stand of cultivar VRG1, a new grape selection under study. Berries were rinsed with tap water, surface sterilized with a sodium hypochlorite solution (1% available chloride) for 3 min, and rinsed four times with sterile distilled water. Sterilized tissues from the rot area were aseptically cut into 5 × 5-mm pieces, finely minced in a few drops of sterile distilled water, streaked on 5% sucrose-nutrient agar (SNA) medium, and incubated in the dark at 25°C for up to 48 h. The “levan-type” colonies, the only ones isolated exclusively from symptomatic berries, were subcultured onto sucrose nutrient agar medium (SNA) to obtain pure colonies. These were streaked on King's medium B (King et al. 1954), and fluorescence was observed under ultraviolet (UV) light (254 nm) after 48 h at 25°C. Pure colonies were stored in nutrient agar (NA) slant tubes at 4°C and in 20% glycerol at –80°C for further use. Colonies

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grown for 48 h onto NA slant tubes were used for biochemical and pathogenic assays.

Biochemical and nutritional tests. The selected bacterial isolates Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro (DiSSPA; Bari, Italy)_Pss_20, DiSSPA_Pss_21, DiSSPA_Pss_22, and DiSSPA_Pss_23, showing “levan-type” colonies and representative of the populations present in the four sampled vineyards, and the type strain of *P. syringae* pv. *syringae* National Collection of Plant Pathogenic Bacteria (NCPB; Harpenden, United Kingdom) 281 were characterized by LOPAT test for levan production from sucrose (L), oxidase activity (O), pectolytic activity on potato tubers, arginine dihydrolase (A), and tobacco hypersensitivity (TH) (Lelliott and Stead 1987). The same isolates were also subjected to GATTA tests consisting of gelatin hydrolysis (G), aesculin hydrolysis (A), tyrosinase activity (T), and tartrate utilization (Ta) (Latorre and Jones 1979). Their capability in 2-keto gluconate production, nitrate reduction, acid production from sucrose, arbutin hydrolysis,

and utilization of inositol, sorbitol, erythritol, L(–)lactate, and D(–)tartrate as carbon sources was ascertained according to Lelliott and Stead (1987) and Schaad et al. (2001).

Molecular assay. Genomic DNA was extracted from a 24-h Luria Bertani (LB) culture according to the protocol described by Marmur (1961) and analyzed for the partial sequences of four core genes (*16S ribosomal DNA* [*rDNA*], *gyrB*, *rpoB*, and *rpoD*). The primer pairs for the target sequences are listed in Table 1. The isolates DiSSPA_Pss_20, DiSSPA_Pss_21, DiSSPA_Pss_22, DiSSPA_Pss_23, and NCPB 281 were used in the experiment.

The polymerase chain reaction (PCR) mixture contained 5 µl of 10× LA Taq buffer, 5 µl of 25 mM MgCl₂, 8 µl of 10 mM dNTPs mixture, 200 nM each primer (Table 1), 0.5 µl of Takara LA Taq, 3 µl of template DNA (50 ng µl⁻¹), and ultrapure water up to 50 µl. Amplifications were carried out in a MyCycler Thermocycler (Bio-Rad Laboratories) using the following PCR conditions: 94°C for 3 min followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min with a final extension at 72°C for

Table 1. Primer used

Primer name	Sequence (5' to 3')	Target	References
FD1	CCGAATTCGTCGACAACAGAGTTTATCCTGGCTCAG	16S ribosomal DNA	Weisburg et al. 1991
RD1	CCCGGATCCAAGCTTAAGGAGGTGATCCAGCC		
Pss_gyrBF	AAGTATCCGGTGGTTGCAC	<i>gyrB</i>	
Pss_gyrBR	CAGACCTTCCTGCTCGATGT		
LAPS	TGGCCGAGAACCAGTTCGCGT	<i>rpoB</i>	Whitelaw-Weckert et al. 2011
LAPS27	CGGCTTCGTCCAGCTTGTTTACG		
Pss_rpoDF	AGAGGCATCCGTGAAGTGAT	<i>rpoD</i>	
Pss_rpoDR	CATGATGGCGCGTTCCTG		

Table 2. Description of *Pseudomonas* species used in the phylogenetic analyses

Species (strain)	Host	Location
<i>Pseudomonas amygdali</i> pv. <i>morsprunorum</i> (2341)	<i>Prunus cerasus</i>	Hungary
<i>Pseudomonas brassicacearum</i> (DF41)	<i>Brassica napus</i>	Manitoba, Canada
<i>Pseudomonas cichorii</i> (JBC1)	<i>Glycine max</i>	South Korea
<i>Pseudomonas corrugata</i> (RM1-1-4)	<i>Brassica napus</i>	Graz, Austria
<i>Pseudomonas fluorescens</i> (L228)	<i>Miscanthus giganteus</i>	Carlow, Oakpark, Ireland
<i>Pseudomonas marginalis</i> (ICMP 11289)	<i>Actinidia deliciosa</i>	New Zealand
<i>Pseudomonas savastanoi</i> pv. <i>phaseolicola</i> (1448A)	<i>Phaseolus vulgaris</i>	Ethiopia
<i>Pseudomonas syringae</i> pv. <i>actinidiae</i> (ICMP 9617)	<i>Actinidia deliciosa</i>	New Zealand
<i>Pseudomonas syringae</i> pv. <i>aptata</i> (ICMP459)	<i>Beta vulgaris</i>	U.S.A.
<i>Pseudomonas syringae</i> pv. <i>atrofaciens</i> (ICMP4394)	<i>Triticum aestivum</i>	Auckland, New Zealand
<i>Pseudomonas syringae</i> pv. <i>cerasicola</i> (ICMP17524)	<i>Prunus x yedoensis</i>	Japan
<i>Pseudomonas syringae</i> pv. <i>lapse</i> (ATCC 10859)	<i>Triticum aestivum</i>	Unknown
<i>Pseudomonas syringae</i> pv. <i>persica</i> (NCPB 2254)	<i>Prunus persica</i>	France
<i>Pseudomonas syringae</i> pv. <i>pisi</i> (PP1)	<i>Pisum sativum</i>	Japan
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (642)	<i>Plantago rugelii</i>	Blacksburg, VA, U.S.A.
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (1212)	<i>Pisum sativum</i>	United Kingdom
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (2339)	<i>Prunus avium</i>	Hungary
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (A2)	<i>Pyrus calleryana</i>	Unknown
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (Alf3)	<i>Medicago sativa</i>	Laramie, WY, U.S.A.
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (B64)	<i>Triticum aestivum</i>	Unknown
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (B301D)	<i>Pyrus communis</i>	United Kingdom
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (B728a)	<i>Phaseolus vulgaris</i>	Wisconsin, U.S.A.
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (CRAFRU11)	<i>Corylus avellana</i>	Italy
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (DAR 73915)	<i>Vitis vinifera</i> (leaves)	Australia
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (DAR 77819)	<i>Vitis vinifera</i> (berry)	Tumbarumba, Australia
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (DAR 77820)	<i>Vitis vinifera</i> (rachis)	Tumbarumba, Australia
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (HR-IW 7924)	<i>Prunus cerasus</i>	United Kingdom
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (HS191)	<i>Panicum miliaceum</i>	Australia
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (MW844)	<i>Vitis vinifera</i> (rachis)	Tumbarumba, Australia
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (MW953)	<i>Vitis vinifera</i> (shoot)	Tumbarumba, Australia
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (ICMP3023)	<i>Syringa vulgaris</i>	United Kingdom
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (SM)	<i>Triticum aestivum</i>	U.S.A.
<i>Pseudomonas syringae</i> pv. <i>theae</i> (NCPB 2598)	<i>Camellia sinensis</i>	Japan
<i>Pseudomonas syringae</i> pv. <i>tomato</i> (DC3000)	<i>Lycopersicon esculentum</i>	U.S.A.
<i>Pseudomonas tolaasii</i> (2192T)	<i>Agaricus bisporus</i>	Reading, United Kingdom

10 min. All PCR products were purified by using PCR Kleen Purification Spin Columns (Bio-Rad Laboratories) and custom sequenced (Genewiz) in both directions (*16S rDNA*: 900 bp) or a single direction for the others (*gyrB*: 500 bp; *rpoB*: 844 bp; and *rpoD*: 431 bp).

Amplicons of the *16S rDNA* sequences of all of the isolates used (DiSSPA_Pss_20 to 23; GeneBank accession numbers MK156155, MK156156, MK15615, and MK156158, respectively) were previously aligned in both directions and self-compared using the SeqMan Pro software (DNASTAR, Inc.) before their comparison with sequences deposited in GenBank (<https://www.ncbi.nlm.nih.gov/>). Identification was completed by alignment and self-comparison of the *gyrB* (GeneBank accession numbers MK165100, DiSSPA_Pss_20; MK165101, DiSSPA_Pss_21; MK165102, DiSSPA_Pss_22; and MK165103, DiSSPA_Pss_23), *rpoB* (MK165104, DiSSPA_Pss_20; MK165105, DiSSPA_Pss_21; MK165106, DiSSPA_Pss_22; and MK165107, DiSSPA_Pss_23), and *rpoD* (MK165108, DiSSPA_Pss_20; MK165109, DiSSPA_Pss_21; MK165110, DiSSPA_Pss_22; and MK165111, DiSSPA_Pss_23) sequenced genes with those from GenBank (<https://www.ncbi.nlm.nih.gov/>). The *16S rDNA*, *gyrB*, *rpoB*, and *rpoD* gene sequences of *Pseudomonas* sp. (Table 2), directly downloaded from The Pseudomonas Genome Database (www.pseudomonas.com), were aligned and trimmed with SeqMan Pro Software (DNASTAR, Inc.) to obtain concatenated sequences. Phylogenetic analyses using *16S rDNA-gyrB-rpoB-rpoD* concatenated sequences and *rpoD* sequences were conducted in MEGA6 (Tamura et al. 2013) using the unweighted pair group method with arithmetic mean (UPGMA). Similarly, the *P. syringae* pv. *syringae* isolated from grape was

compared using *ropB* sequences, because they were the only ones available in GenBank.

Antimicrobial activity. The syringomycin and syringopeptin production of DiSSPA_Pss_20, DiSSPA_Pss_21, DiSSPA_Pss_22, DiSSPA_Pss_23, and NCPPB 281 was assayed according to Gross and DeVay (1977). Aliquots (5 μ l) of bacterial suspension (about 10^8 CFU ml⁻¹) were spotted onto potato dextrose agar (PDA) and maintained at 25°C. After 4 days, plates were oversprayed with either a dense arthrospore suspension of the fungus *Geotrichum candidum*, known for its sensitivity to syringomycin and insensitivity to syringopeptin (Grgurina et al. 1996; Lavermicocca et al. 1997), or a suspension of *Bacillus megaterium*, a bacterium highly sensitive to syringopeptin and less sensitive to syringomycin (Lavermicocca et al. 1997). For each test, three replicated plates were used. The suspensions of *G. candidum* and *B. megaterium* were obtained from colonies grown on PDA for 4 days at 25°C. Inhibition was recorded after 48 h at 25°C in the dark.

Pathogenicity and host range tests. All *P. syringae* pv. *syringae* isolates were tested for their ability to elicit a hypersensitivity reaction by infiltration in cultivar Samsun tobacco leaves. The pathogenicity of isolates DiSSPA_Pss_20, DiSSPA_Pss_21, DiSSPA_Pss_22, and DiSSPA_Pss_23 was proven by inoculating detached table grape berries, unripe green lemon fruits, and lilac leaves (Klement et al. 1990; Lelliott and Stead 1987). Two-day-old bacterial cells grown on SNA at 25°C were suspended in sterile distilled water and adjusted to an appropriate final concentration as detailed below. Detached berries, fresh lemons, and lilac leaves were also inoculated

Fig. 1. A, Symptoms on *Pseudomonas* grape bunch rot on cultivar VRG1 in the field. B, Symptoms on VRG1 berries 20 days after inoculation (DAI) with Pss_20 (upper row) and water as negative control (lower row). C, Pathogenicity assay on lilac leaves 10 DAI with Pss_20 (1), National Collection of Plant Pathogenic Bacteria (NCPPB)-type strain (2), and water as negative control (3). D, Black pit lesions on lemon fruit 7 DAI with NCPPB-type strain (1), Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro_Pss_20 (2), and water as negative controls (3 and 4).

with the type strain NCPPB 281 and sterile distilled water as positive and negative controls, respectively.

Ten surface-sterilized (1% NaClO solution for 3 min) berries of four different table grape cultivars (Red Globe, cultivar Sweet Globe, VRG1, and cultivar VRG2; VRG1 and VRG2 are two new selections currently under evaluation) were prick inoculated with a hypodermic needle, with a droplet of bacterial suspension (10^8 CFU ml⁻¹) placed at the inoculation site. Inoculated berries were maintained at high humidity on moist filter paper in a sterile sealed plastic container at room temperature for 20 days.

Lemons were surface sterilized by dipping into 2% NaClO solution for 2 min, and then, they were rinsed with sterile distilled water. Four punctures were made on the fruits (1-mm wide and 3-mm deep) with a sterile hypodermic needle; then, 20 µl of bacterial suspension (10^6 CFU ml⁻¹) was placed on each lesion (Lelliott and Stead 1987). Four lemons for each condition were used. Inoculated fruits were placed on moist filter paper in a sterile sealed plastic box and incubated at 25°C for 1 week.

Detached lilac leaves were wounded according to Ivanović et al. (2017) with a sterile hypodermic needle by pricking at three to four points the leaf midrib and areas on the wing of the petiole of fully expanded leaves and placing a 10-µl drop of bacterial suspension (10^6 CFU ml⁻¹). Inoculated leaves and petioles were placed in sterile plastic bags and maintained for 10 days at room temperature (never exceeding 21°C) and 70 to 80% humidity. Reactions were assessed 7, 14, and 20 days postinoculation (DPI). All of the experiments were repeated twice.

Results

Unexpected and new soft and watery spots on berries (Fig. 1A) evolving in necrotic spots were observed on bunches of Red Globe and VCG1. The season in which these symptoms appeared was particularly humid during blossoming and then, hot with broad night/day thermal excursions. Only “levan-type” bacterial colonies were isolated from all symptomatic grape berries. All isolates were gram negative and on King’s medium B, produced a green to blue diffusible pigment fluorescent under UV light, such as the NCPPB

281-type strain. Data on biochemical, physiological, and nutritional characterization as well as data on antimicrobial activity and pathogenicity are summarized in Figure 1 and Table 3. The four representative grape berry isolates were positive for levan; were negative for oxidase, potato soft rot, and arginine dihydrolase; and elicited a hypersensitive reaction on tobacco leaves. Furthermore, they produced gelatin and aesculin hydrolysis but did not produce tyrosinase, and unlike the NCPPB 281-type strain, they were able to utilize L(+)-tartrate (GATTa[±]). Unlike NCPPB 281-type strain, the grape isolates did not utilize L-lactic acid. All isolates, however, hydrolyzed arbutin, produced acidity from sucrose, did not reduce nitrates, did not produce 2-ketogluconate, and were able to use sorbitol, inositol, and erythritol but not D-tartrate.

The antimicrobial activity was assayed versus *B. megaterium* and *G. candidum* on PDA. The grape *Pseudomonas* isolates strongly inhibited *B. megaterium* but were ineffective against *G. candidum*; this indicated the ability to produce syringopeptin but not syringomycin, unlike the NCPPB 281-type strain, which inhibited both microorganisms.

To fulfill identification of the four *P. syringae* pv. *syringae* isolates (DiSSPA_Pss_20 to 23), the partial gene sequences of *16S rDNA*, *gyrB*, *rpoB*, and *rpoD* were analyzed, and the self-identity between *P. syringae* pv. *syringae* strains was 100% (e-value 0.0, coverage 100%). For all of them, the nucleotide blast analysis of the *16S rDNA*, *gyrB*, *rpoB*, and *rpoD* gene sequences showed identities of 99 to 100% (e-value 0.0, coverage 100%) with different *P. syringae* pathovars; 99 to 100% (e-value 0.0, coverage 100%) with *P. syringae* pv. *syringae* and *P. syringae* pv. *aptata*; 99% (e-value 0.0, coverage 98%) with *P. syringae* pv. *syringae*, *P. syringae* pv. *lapsa*, and *P. syringae* pv. *atrofaciens*; and 98% (e-value 0.0, coverage 99 to 100%) with *P. syringae* pv. *syringae*, *P. syringae* pv. *aptata*, *P. syringae* pv. *lapsa* and *P. syringae* pv. *atrofaciens*, respectively. Generally, lower identities values were observed for other *P. syringae* pathovars as well as other *Pseudomonas* species.

The similarity of all the grape berry isolates DiSSPA_Pss_20-23 and Pss strains from grape and other hosts was confirmed also by phylogenetic analysis (Fig. 2).

Table 3. Biochemical, pathological, and antimicrobial characters of the bacterial grape berry isolates and the *Pseudomonas syringae* pv. *syringae*-type strain used^a

Tests	Grape berry isolates				<i>Pseudomonas syringae</i>
	DiSSPA_Pss_20	DiSSPA_Pss_21	DiSSPA_Pss_22	DiSSPA_Pss_23	pv. <i>syringae</i> -type strain NCPPB 281
Gram	–	–	–	–	–
Fluorescence	+	+	+	+	+
Levan (L)	+	+	+	+	+
Oxidase (O)	–	–	–	–	–
Potato soft rot (P)	–	–	–	–	–
Arginine dihydrolase (A)	–	–	–	–	–
Tobacco hypersensitivity (T)	+	+	+	+	+
Gelatin hydrolysis (G)	+	+	+	+	+
Aesculin hydrolysis (A)	+	+	+	+	+
Tyrosinase activity (T)	–	–	–	–	–
L(+)-tartrate (Ta)	+	+	+	+	–
Utilization of					
L-lactate	–	–	–	–	+
D-tartrate	–	–	–	–	–
Sorbitol	+	+	+	+	+
Inositol	+	+	+	+	+
Erythritol	+	+	+	+	+
Arbutin hydrolysis	+	+	+	+	+
Nitrate reduction	–	–	–	–	–
2-Ketogluconate production	–	–	–	–	–
Pathogenicity on					
Lemon fruit	+	+	+	+	+
Lilac leaves	+	+	+	+	+
Inhibition of					
<i>Bacillus megaterium</i>	+	+	+	+	+
<i>Geotrichum candidum</i>	–	–	–	–	+

^a DiSSPA, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Bari, Italy; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.

All tested isolates were pathogenic to grape. On all VRG1 grape berries, water-soaked lesions and necrosis of tissues were observed around the inoculation site 10 DPI with all of the grape isolates DiSSPA_Pss_20 to 23 and the NCPPB-type strain, although symptoms were generally less severe than those observed in the field. Instead, on VRG2 and Sweet Globe, which were symptomless in the field, the symptoms appeared only on 10 to 20% of the inoculated berries. Reisolated colonies exhibited the same morphological and biochemical traits as those used for inoculation.

Lemon fruits inoculated with the grape *Pseudomonas* isolates showed black pit lesions within 1 week that were less extensive but comparable with those observed on fruits inoculated with NCPPB 281-type strain. Lilac leaves reacted to the grape *Pseudomonas* isolates and NCPPB 281-type strain with the production of a water-soaked or darkened area, expanding along the midvein. No symptoms were observed in lemons and lilac leaves used as controls.

Discussion

A large number of studies on *P. syringae* have been conducted, because this bacterium is used as a model for studying plant-microbe interactions (Alfano and Collmer 2004; Hulin et al. 2018; Xin and He 2013). *P. syringae* is known to be responsible for a large number of disease emergencies (Bartoli et al. 2015a, b; Cai et al. 2011; Diallo et al. 2012; Mohr et al. 2008; Morris et al. 2013; Yan et al. 2008). In fact, considering only this century, new diseases caused by *P. syringae* have been reported on ≥ 20 different species of woody plants (Elena et al. 2011; Lamichhane et al. 2014; Santini et al. 2013). In the European and Mediterranean Plant Protection Organization (EPPO) region, bacterial canker of kiwifruit caused by *P. syringae*

pv. *actinidiae* was first detected in central Italy in 1992 (Scortichini 1997), where it remained sporadic and with a low incidence for 15 years. However, it became very severe in 2007 and 2008, particularly in the Lazio region, where it induced heavy economic losses; then, it moved to other kiwifruit-producing regions in the Mediterranean area.

On grapevine, *P. syringae* has been reported as the cause of extensive damage, including necrosis of leaf tissues, shoots, tendrils, and rachises (Hall et al. 2002; Klingner et al. 1976); bark necrosis (Cugusi et al. 1986); and generic bacteriosis (Samedov et al. 1988). *P. syringae* pv. *syringae* has been ascertained to be responsible for bacterial inflorescence rot in Australia (Whitelaw-Weckert et al. 2011). It was proved to be a motile inhabitant of plant surfaces, soil, and water, and it overwintered in wood; after grapevine dormancy, it can spread across wet surfaces of emerging shoots, leaves, and inflorescences (Whitelaw-Weckert et al. 2011).

As proven by the fulfillment of Koch's postulates, this is the first report of *Pseudomonas* bunch rot of grapevine caused by *P. syringae* pv. *syringae* according to the results of biochemical, physiological, nutritional, antimicrobial activity, and pathogenicity tests and sequencing of *16S rDNA*, *gyrB*, *rpoB*, and *rpoD* genes. Likely, *Pseudomonas* bunch rot was favored by seasonal weather that was particularly humid during blossoming and then hot with broad night/day thermal excursions.

According to the LOPAT test, the four representative grape berry isolates proved to belong to the LOPAT group Ia (sensu Lelliott et al. 1966), which includes *P. syringae* pathovars. They, unlike the NCPPB 281-type strain and the *P. syringae* pv. *syringae* strains, were able to utilize L(+)-tartrate (GATTA[±]) but did not utilize L-lactic acid.

Fig. 2. Phylogeny of isolates Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro_Pss_20 to 23 with other *Pseudomonas syringae* pv. *syringae* strains and *P. syringae* pathovars by using **A**, concatenated 16S ribosomal DNA, *gyrB*, *rpoB*, and *rpoD* gene sequences and **B**, only *rpoD* gene sequences. Dendrograms was generated by the unweighted pair group method with arithmetic mean (UPGMA). *Pseudomonas marginalis* (ICMP11289), *Pseudomonas cichorii* (JBC1), *Pseudomonas tolaasii* (2192T), *Pseudomonas fluorescens* (L228), *Pseudomonas corrugata* (RM1-1-4), and *Pseudomonas brassicacearum* (DF41) were used as outgroups. Percentage bootstrap values of $>50\%$ (from 1,000 replicates) are indicated at the nodes. *Probably synonyms of *P. syringae* pv. *syringae* (Whitelaw-Weckert et al. 2011).

Generally, the LOPAT and GATTA testing schemes are used to separate *P. syringae* pv. *syringae* from other *Pseudomonas* species and *P. syringae* pathovars, also considering that a few mismatches can sometimes occur (Scheck et al. 1997).

The differences in *16S rDNA* sequences are always very small, and although they are effective for the identification of genera, they may be not be enough to distinguish strains of the same genus (Yamamoto and Harayama 1995). Thus, three additional gene sequences for the isolate DNA were examined according to previous works on *Pseudomonas* pathovars identification (Sawada et al. 1999; Whitelaw-Weckert et al. 2011). Genome-wide analyses of the effector- and toxin-encoding genes were used to examine the phylogenetics and evolution of pathogenicity among diverse strains of *P. syringae* causing bacterial canker of cherry (*Prunus avium*), including pathovars *P. syringae* pv. *morsprunorum* races 1 and 2, *P. syringae* pv. *syringae*, and *P. syringae* pv. *avii*. Phylogenetic analyses of the Italian grape berry *P. syringae* pv. *syringae* strains revealed that all were claded and interspersed among strains from other host species, providing an example of convergent evolution of pathogenicity (Hulin et al. 2018).

In pathogenicity tests, Red Globe and the new grapevine selection VRG1 proved to be much more susceptible than Sweet Globe and VRG2. Likewise, the grapevine bacterial strains differed slightly from the NCPBP 281-type strain. Pss_20 was a high syringopeptin producer, but in the experimental condition adopted, it was unable to produce syringomycin, results that are in accordance with the work by Latorre and Jones (1979). Syringomycins and syringopeptins are two classes of necrosis-inducing lipodepsipeptide toxins characterizing *P. syringae* pv. *syringae* (Scholz-Schroeder et al. 2001), and they are produced in infected plant tissues (Fogliano et al. 1999; Grgurina et al. 1997). They play roles as virulence factors in plant diseases (Scholz-Schroeder et al. 2001). Although both elicit necrotic symptoms in host tissues and are highly phytotoxic, syringomycin was 30 times more active in antifungal activity assays, and it was also shown to reduce stomata apertures in leaves of *Vicia faba* (Mott and Takemoto 1989). However, it has been shown that not all strains produce such compounds (Gross and De Vay 1977; Scortichini et al. 2003; Zeller et al. 1997). Different authors instead have shown that syringopeptin is more phytotoxic than syringomycin (Dalla Serra et al. 1999; Hutchison and Gross 1997; Iacobellis et al. 1992a; Scholz-Schroeder et al. 2001), and *P. syringae* pathovars are known to produce a wide spectrum of secondary metabolites exhibiting phytotoxic capabilities (Bender et al. 1999; Gross 1991). Thus, the pathogenic contribution of a phytotoxin depends on the specific host-pathogen interaction. In addition to their phytotoxic effects, these lipopeptides have prominent antibiotic properties exhibiting differential antifungal and antibacterial activities against a variety of microorganisms (Iacobellis et al. 1992a, b; Lavermicocca et al. 1997). They are strongly inhibitory to gram-positive bacteria, particularly *Bacillus* spp. (Lavermicocca et al. 1997). The use of biocontrol agents in the management of bunch rots is increasing (Rotolo et al. 2018). Because many of them are lipopeptide producers, it can be supposed that, in some way, they can increase the virulence and/or the fitness of *P. syringae* pv. *syringae*. This concern is heightened by changes in climate and commercial networks that could intensify emerging epidemics of plant disease in areas well known for their crop vocation. The characterization of the metabolites associated with the bunch rot *P. syringae* pv. *syringae* type will be improved, and studies on the relationship between grapevine cultivars and *P. syringae* pathovars are in progress.

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